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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	US APPLICATION NO. (If known, see 37 CFR 1.5)
442P090	10/030294

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/04261	28 June 2000	29 June 1999

TITLE OF INVENTION: GENE ENCODING PROMOTER REGION OF TUMOR SUPPRESSOR GENE P51 AND USE THEREOF

APPLICANT(S) FOR DO/EO/US: Toshiyuki Sakai, Shigehide Kagaya, Takamichi Sato, Yoshikazu Sukenaga and Hideji Fujii

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [X] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [X] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information:
    1. Disk containing sequence listing; and 27-Page printed sequence listing.
    2. Copy of the International Search Report;
    3. Copy of Form PCT/IB/308; and
    4. Copy of Form PCT/IB/304.

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21. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	15 - 20 =	0	x $18.00	$ 0.00	
Independent claims	2 - 3 =	0	x $84.00	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$ 280.00	
			TOTAL OF ABOVE CALCULATIONS =	$ 1,170.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$ 0	
			SUBTOTAL =	$ 1,170.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$ 0	
			TOTAL NATIONAL FEE =	$ 1,170.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$ 40.00	
			TOTAL FEES ENCLOSED =	$ 1,210.00	
			Amount to be refunded:	$	
			charged:	$	

a. ☒ A check in the amount of $ 1,170.00 & 40.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0930 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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DESCRIPTION

GENE ENCODING PROMOTER REGION OF TUMOR SUPPRESSOR GENE P51 AND USE THEREOF

TECHNICAL FIELD

The present invention relates to a gene encoding the promoter region of a protein p51 which is capable of inducing cell death, suppressing cell growth, etc. and a gene encoding the 5'-untranslated region of p51. The present invention also relates to a series of uses of these genes.

BACKGROUND ART

The tumor suppressor gene p53 encodes a protein having a variety of functions such as the induction of apoptosis, cell cycle arrest, the repairing of DNA damages, and the like. It has also been shown that p53 is a transcription factor which regulates the expression of various proteins. Currently this protein is believed to play a central role in the control of cell growth. It has also been reported that p53 mutation is observed in about half of tumor cells which is mainly responsible for abnormal growth and resistance against anti-cancer agents. Although no proteins were known that have a p53-like structure and function until very recently, but two novel p53-like molecules were recently reported. One

is p73 (Cell, 90:809-819, 1997), and the other is p51 (Nature Medicine, 4:839-843, 1998). Concerning p51, a protein encoded by the same gene is reported as p63 (Molecular Cell, 2:305-316, 1998). Structurally p51 is very similar to p53, and like p53 it is capable of activating the transcription of a cell cycle progression suppressor protein p21, suggesting that it is homologous to p53 functionally as well. Furthermore, it is reported that p51, as is p53, is capable of inducing cell death, suppressing cell growth, and the like. It has been shown, however, that p51 is different from p53 in that no mutation in p51 has been observed in tumor cells. It is being elucidated that p51 is expressed in very limited organs such as muscle cells.

DISCLOSURE OF THE INVENTION

It is an object of the present invention to identify the gene encoding the promoter region of a tumor suppressor gene p51 and the gene encoding the 5'-untranslated region of p51, thereby to provide a method of cloning these genes, a method of screening novel drugs using these genes, and a novel method of gene therapy using these genes, and the like.

After intensive and extensive research to find a gene fragment containing the p51 promoter in a human genomic library, the inventor of the present invention has identified a gene sequence having the p51

promoter region, and a gene sequence encoding the 5'-untranslated region of p51, and thereby has completed the present invention.

Thus, a human genome fragment containing a same sequence as cDNA complementary to p51 mRNA was screened and isolated by the plaque hybridization screening method. Furthermore, the base sequence of this gene fragment was determined to confirm that it contains regions that are estimated to be the p51 promoter region and the 5'-untranslated region of p51. A plasmid was also constructed in which the gene fragment is ligated to the luciferase reporter gene, and it was then introduced into a human cell line to construct a transformant. By further analyzing this transformant, it was found out that said gene can work as a promoter. Furthermore, there was found a method of screening an agent that acts on the p51 promoter using transformant, and a possibility that a pharmaceutical agent that enhances this promoter activity acts as a therapeutic agent such as an anti-cancer agent for diseases in which abnormality in p53-dependent apoptosis is involved.

Thus, the present invention relates to a gene encoding the p51 promoter region shown in the following (1), (2), (3), (4), (5), or (6):

(1) DNA that encodes the p51 promoter region having the base sequence as set forth in SEQ ID NO: 1 of the sequence listing;

(2) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in the base sequence as set forth in SEQ ID NO: 1 of the sequence listing, and that has p51 promoter activity;

(3) DNA that hybridizes to the base sequence as set forth in SEQ ID NO: 1 of the sequence listing under a stringent condition, and that has p51 promoter activity;

(4) DNA that has the base sequence as set forth in SEQ ID NO: 2 of the sequence listing, and that encodes the p51 promoter region and the 5'-untranslated region of p51;

(5) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing, and that has p51 promoter activity; and

(6) DNA that hybridizes to the base sequence as set forth in SEQ ID NO: 2 of the sequence listing under a stringent condition, and that has p51 promoter activity.

The present invention also relates to a gene encoding the 5'-untranslated region of p51 shown in the following (7), (8), or (9):

(7) DNA that has a base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing;

(8) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in a base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing, and that has a function similar to that of DNA in the above (7); and

(9) DNA that hybridizes to DNA comprising the base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing under a stringent condition, and that has a function similar to that of DNA in the above (7).

The present invention further relates to a recombinant plasmid comprising the gene in the above (1) to (6).

The present invention further relates to a transformant or a transductant comprising the above recombinant plasmid.

The present invention further relates to nucleic acid probes comprising all or parts of the base sequence of the gene in the above (1) to (9).

The present invention further relates to a method of cloning p51 promoter by hybridization using the above nucleic acid probe.

The present invention further relates to a DNA sequence that is an antisense to all or part of the base sequence of the gene in the above (1) to (9), and that can modify the function of p51 promoter activity.

The present invention further relates to a

RNA sequence that is an antisense to all or part of the base sequence of the gene in the above (1) to (9), and that can modify the function of p51 promoter activity.

The present invention further relates to a method of screening drugs that act on the p51 promoter using the above transformant or the above transductant.

The present invention further relates to a compound that enhances or inhibits the expression of p51 gene, said compound being selected by the above screening method.

The present invention further relates to a pharmaceutical formulation comprising the above DNA or the above RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing a restriction map of pGL2-neo/p51 promoter. In the figure, amp represents an ampicillin-resistant gene, neo represents a neomycin-resistant gene, and luc represents the luciferase gene. The name of a restriction enzyme that has a * mark on the left means that the vector can be only cleaved at the point.

Figure 2 shows an analysis in which the amount of p51 expressed was analyzed using the technique of Northern blotting after a cell line HCT116 derived from colon cancer, was subjected to various stimulations. In each lane is blotted 20 μg of RNA from: lane 1, 2: cells treated with taxol (1 μg/ml, 10

μg/ml), lane 3, 4: cells treated with trichostatin A (0.1 μg/ml, 1 μg/ml), lane 5, 6: cells treated with radiation (50 J/m^2, 100 J/m^2), lane 7, 8: cells treated with vitamin D_3 (10 nM, 100 nM), lane 9, 10: cells treated with vincristine (1 μg/ml, 10 μg/ml), and lane 11, 12: cells treated with wortmannin (0.1 μm, 1 μm). A band that reacts with the p51 probe is observed at the position indicated by an arrow.

Figure 3 shows a cell line HCT116 derived from colon cancer, into which 2 μg each of pGL2-neo/p51 promoter or pGL2-neo vector was introduced and 24 hours later, 0 or 1 μg/ml trichostatin A (TsA) was added and was further cultured for 24 hours. By determining the luciferase activity in these cells, the promoter activity of the isolated gene fragments was examined. As a negative control cell, cells into which 2 μg of pcDNA3/lacZ was introduced were used. As a result, it was found that an extract of the pGL2-neo/p51 promoter-introduced cells exhibits a significantly higher fluorescence intensity than that of the pGL2-neo-introduced cells. The administration of trichostatin A further enhanced the chemilumicescence intensity in the extract of the pGL2-neo/p51 promoter-introduced cells.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will now be explained in further details below.

(1) Gene having the p51 promoter region of the present

invention

(1-1) In order to isolate a gene having the p51 promoter region of the present invention, a human genomic DNA library is constructed, and from the library, using part of the sequence described in the above-mentioned article in Nature Medicine, a cDNA molecule that hybridizes to a cDNA complementary to the 5'-untranslated region of p51 mRNA can be isolated and cloned resulting in the isolation of a gene encoding the p51 promoter region.

The base sequence of a gene encoding the p51 promoter region of the present invention is as set forth in SEQ ID NO: 1. In this gene sequence, TATA boxes have been observed in the bases in positions from 5630 to 5636 and in positions from 5659 to 5665 of the above same sequence listing. They are located at 47 bases and 18 bases upstream of a gene sequence (GenBank AB016072) reported as the sequence of p51A protein mRNA (p51AmRNA), respectively. Furthermore, the binding site of MEF2 known to be a muscle cell-specific transcription factor is located in the bases at positions from 1211 to 1220 of the same sequence listing. In a search of the database for genes having a homology with the gene encoding The p51 promoter region as set forth in SEQ ID NO: 1 of the sequence listing, no sequences had a homology of 30% or higher over the entire length.

Furthermore, for the purpose of confirming the function of the gene having the base sequence as

set forth in SEQ ID NO: 1 of the sequence listing, as shown in the following Example, a plasmid was constructed in which a gene having the base sequence as set forth in SEQ ID NO: 1 of the sequence listing and the luciferase reporter gene were ligated, which was then expressed in a cultured human cell line to construct a transformant. The transformant significantly expressed the luciferase reporter gene as compared to the control group. Furthermore, a simple screening using the transformant led to the discovery of the p51 promoter activating ability in trichostatin A. One μg/ml of trichostatin A (manufactured by Wako Pure Chemical Industries, Ltd.) transactivates p51 in the cultured human cell line, and it was confirmed under this condition that the promoter activity of the gene having the isolated p51 promoter region was further remarkably enhanced. Thus, these results demonstrate that the gene having the base sequence as set forth in SEQ ID NO: 1 of the sequence listing has a function as a p51 promoter.

(1-2) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in the base sequence as set forth in SEQ ID NO: 1 of the sequence listing, and that has p51 promoter activity, is also encompassed in the scope of the present invention. The deletion, substitution, or addition of bases is to a degree that does not substantially affect the structure and function of the entire

promoter. The degree of deletion, substitution, or addition of these bases is acceptable when it has p51 promoter activity and it has a homology with the original base sequence of 80% or higher, preferably 90% or higher, and more preferably 99% or higher.

(1-3) DNA that hybridizes to DNA comprising the base sequence as set forth in SEQ ID NO: 1 of the sequence listing under a stringent condition and that has a p51 promoter activity is also encompassed in the scope of the present invention. Such hybridizing DNA mutants include DNA sequences partially altered by the mutation, deletion, ligation etc. of DNA fragments by site-directed mutagenesis, mutagen-treated random mutation, cleavage with restriction enzymes. The degree to which these DNA mutants hybridize to the coding gene as set forth in SEQ ID NO: 1 is a stringent condition: for example, the above membrane is incubated in a hybridization solution (50 mM Tris-HCl, pH 7.5, 1 M sodium chloride, 1% sodium dodecylsulfate, 10% dextran sulfate, 0.2 mg/ml yeast RNA, 0.2 mg/ml salmon sperm DNA) at 65°C for one hour as prehybridization, then a radioisotope-labeled cDNA fragment is added to one million dpm/ml in terms of the amount of radioisotope and incubated at 65°C for 16 hours as hybridization, and subsequently the membrane is washed in a 2 x SSC solution (300 mM sodium chloride, 30 mM trisodium citrate) containing 0.1% sodium dodecylsulfate at 65°C for 30 minutes, followed by autoradiography analysis to

confirm hybridization on an X-ray film.

(1-4) DNA that is shown in SEQ ID NO: 2 of the sequence listing and that encodes the p51 promoter region and the 5'-untranslated region of p51 is also encompassed in the scope of the present invention. The base sequence of positions from 5677 to 5960 in the SEQ ID NO: 2 corresponds to DNA encoding 5'-untranslated region. Among them, the base sequence of positions from 5767 to 5960 corresponds to the intron region.

(1-5) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing and that has p51 promoter activity is also encompassed in the scope of the present invention. The deletion, substitution, or addition of bases is to a degree that does not substantially affect the structure and function of the entire promoter. The degree of deletion, substitution, or addition of these bases is acceptable when it has p51 promoter activity and it has a homology with the original base sequence of 80% or higher, preferably 90% or higher, and more preferably 99% or higher.

(1-6) DNA that hybridizes to DNA comprising the base sequence as set forth in SEQ ID NO: 2 of the sequence listing under a stringent condition and that has p51 promoter activity is also encompassed in the scope of the present invention. Such hybridizing DNA mutants include those similar to those exhibited in the

above (1-3). The degree to which these DNA mutants hybridize to the gene as set forth in SEQ ID NO: 2 is such that hybridization is confirmed under a stringent condition similar to the one described in the above (1-3).

(2) Gene encoding the 5'-untranslated region of p51 of the present invention

(2-1) DNA that has a base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing is DNA that encodes the 5'-untranslated region of p51 located downstream of the p51 promoter region, and such DNA is also encompassed in the scope of the present invention. Among them, the base sequence of positions from 5767 to 5960 corresponds to the intron region. Part or all of DNA that encodes the 5'-untranslated region of p51 may be used as a nucleic acid probe, for example, for cloning a gene as described below.

(2-2) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in a base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing, and that has a function similar to that of the DNA in the above (2-1) is also encompassed in the scope of the present invention. The deletion, substitution, or addition of bases is to a degree that it does not substantially affect the structure and function of the DNA. The

degree of deletion, substitution, or addition of these bases is acceptable when it has a function similar to DNA described in the above (2-1) and it has a homology with the original base sequence of 80% or higher, preferably 90% or higher, and more preferably 99% or higher.

(2-3) DNA that hybridizes to DNA comprising the base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing under a stringent condition, and that has a function similar to that of DNA in the above (2-1) is also encompassed in the scope of the present invention. Such hybridizing DNA mutants include those similar to those exhibited in the above (1-3). The degree to which these DNA mutants hybridize to the gene as set forth in SEQ ID NO: 2 is such that hybridization is confirmed under a stringent condition similar to the one described in the above (1-3).

(3) A recombinant plasmid containing a gene having the p51 promoter region of the present invention

By constructing a recombinant plasmid containing a gene having the p51 promoter region of the present invention, it is possible to allow Escherichia coli etc. to stably retain said gene, whereupon vectors used include any commonly used ones such as pBluescript II SK(-). In the examples described below, there is exhibited the pBS/p51 promoter in which a gene having the p51 promoter region has been integrated into

pBluescript II SK(-). After cleaving these plasmids with an appropriate restriction enzyme as needed, they can be ligated to an suitable vector to make plasmids for determining promoter activity. As a plasmid for determining promoter activity, a plasmid such as pGL2 can be used as a vector.

(4) A transformant or a transductant

The above recombinant plasmid can be introduced into an appropriate host to construct a transformant or a transductant. Escherichia coli, yeast, and mammalian cells can be used. A transformant that retains a plasmid for determining promoter activity can be obtained by transforming into an appropriate host a recombinant plasmid that has been integrated into a vector for determining activity as described above. For example, a recombinant plasmid as shown in Figure 1 may be introduced into a cultured mamallian cell to obtain a transformant. A transformant or a transductant may be cultured in an appropriate nutrient culture medium and the amount of the reporter gene expressed in the cell may be measured to determine the promoter activity.

(5) A nucleic acid probe, and gene cloning and detection using it.

In accordance with the present invention, part or all of the gene having the p51 promoter region of the present invention or part or all of the gene encoding the 5'-untranslated region of p51 of the

present invention may be used as a nucleic acid probe for use in gene cloning etc. described below. As a nucleic acid probe comprising part of the gene of the present invention, there may be mentioned a nucleic acid probe comprising an oligonucleotide of 15 nucleotides or more. Said nucleic acid probe may be prepared by ligating the above gene of the present invention or a gene fragment thereof to a suitable vector, which is then introduced into a bacterium, replicated, extracted from the debris of cell homogenate with phenol etc., cleaving with the restriction enzyme that recognizes the site at which the vector is ligated, electrophoresing, and excising the probe from the gel. Said nucleic acid probe may also be prepared, based on the base sequence as set forth in SEQ ID NO: 1 or 2 of the sequence listing, by chemical synthesis using a DNA synthesizer or by a gene amplification technology with polymerase chain reaction (PCR). Said nucleic acid probe may be labeled with a radioisotope or fluorescence to enhance detection sensitivity at the time of use.

Said nucleic acid probe may be used in the cloning method of genes other than the above having a promoter activity or the cloning method of the protein-encoding genes located downstream thereof. Thus, by searching the genome library of various biological tissues by the hybridization method or the PCR method using said nucleic acid probe, it is possible to

isolate genes having a similar function to the gene of the present invention or protein-encoding genes located downstream thereof.

(6) Antisense DNA and antisense RNA

In accordance with the present invention, there may also be provided antisense DNAs and antisense RNAs of genes having the above-mentioned p51 promoter region as well as antisense DNAs and antisense RNAs of genes having the 5'-untranslated region of p51. By introducing these antisense DNAs and antisense RNAs into a cell, the expression of genes encoding p51 can be suppressed or enhanced. As the antisense DNA to be introduced, there can be used, for example, an antisense DNA or part thereof corresponding to SEQ ID NO: 1 or 2 of the sequence listing. An example of said antisense DNA is shown in SEQ ID NO: 3 of the sequence listing. This represents the sequence of the antisense DNA of a gene having the p51 promoter activity of SEQ ID NO: 1 of the sequence listing. As antisense DNA, for example, there can be used a fragment obtained by suitably cleaving part of these antisense DNAs or DNAs synthesized based on the DNA sequences of these antisense DNAs.

As antisense RNA, for example, there can be used antisense RNA or part thereof corresponding to SEQ ID NO: 1 or 2 of the sequence listing. An example of said antisense RNA is shown in SEQ ID NO: 4 of the sequence listing. This represents the sequence of the

antisense RNA of the gene having the p51 promoter activity of SEQ ID NO: 1 of the sequence listing. As antisense RNA, for example, there can be used a fragment obtained by suitably cleaving part of these antisense RNAs or RNAs synthesized based on the RNA sequences of these antisense RNAs. A gene SEQ ID NO: 1 of the sequence listing having the p51 promoter activity or part thereof may be ligated to an appropriate vector, which is then introduced into a bacterium, replicated, extracted from the debris of cell homogenate with phenol etc., which is used as a template to allow RNA polymerase to act in an in vitro transcription system to synthesize RNA to be used. Antisense DNAs and antisense RNAs may be chemically modified so that they are refractory to in vivo decomposition and they can pass through cell membrane. The antisense DNAs and antisense RNAs thus prepared can be used for the treatment of various diseases including malignant tumor.

(7) Drug screening

In accordance with the present invention, there is provided a screening method for novel drugs using the transformant or the transductant of the present invention. For example, an agent that has the p51 promoter region activation activity and that enhances the expression of p51 protein can be discovered by screening drugs that enhance luciferase activity using a cultured cell that has integrated a

recombinant plasmid in which the luciferase reporter gene and said gene have been ligated.

As cultured cells into which a recombinant plasmid is to be introduced, any cultured cell lines that can be passaged can be used including a cultured colon cancer cell line HCT-116. As recombinant plasmids, any vector can be used that contains a reporter gene including pGL2-neo vector in which the neomycin gene has been ligated to the pGL2 plasmid used in Examples. In the construction of a screening system, a cultured cell line is selected into which a vector for screening is introduced by the lipofectin method, etc. By culturing this transformed cell together with the selection drug, cells that contain the vector for screening can only be grown. These cells may be used as they are in screening or may be cloned to be prepared as a single cell line and then used in screening. By adding a sample to these cells and then determining the activity of the reporter protein, agents that regulate the transcription of p51 can be searched. As samples, for example, microbial secondary metabolites may be used, or synthetic compounds may be used. Agents that activates the p51 promoter enhance the production of p51 protein and exhibit a growth suppressing effect on tumor cells, even if p53 is mutated in these cells, and thereby are expected to become novel anti-cancer agents.

(8) Pharmaceutical preparation

The p51's expression is only observed in highly localized tissues such as muscle cells. Thus, it can be used as a gene therapy vector by ligating a gene of interest downstream of the gene having the p51 promoter activity of the present invention, and allowing it to express the gene of interest only in specific tissues. The above antisense DNAs and antisense RNAs can be therapeutic agents for various disease including malignant tumor, also.

Now, the present invention will be specifically explained below. It is to be noted, however, that the present invention is not limited by these examples in any way.

Example 1

Isolation of a novel gene fragment encoding the p51 promoter region

(1-1) Preparation of a probe for screening

A probe for screening a novel gene fragment that encodes the p51 promoter region was prepared. As such a method, the RT-PCR method was used. The experimental procedure is shown below. From a six μg of human muscle-derived RNA (manufactured by OriGene), cDNA was prepared using 200 units of a reverse transcriptase Superscript (manufactured by GIBCO BRL). With this as the template, the amplification of a p51 mRNA-derived gene fragment was attempted by a PCR

method using a primer set shown in SEQ ID NO: 5 and 6 and a primer set shown in SEQ ID NO: 7 and 8. The gene amplified using the former primer set encodes 143 bases of the 5'-untranslated region of p51A mRNA reported until now and 165 bases of the 5'-terminal end of the open reading frame, and the gene amplified using the latter primer set encodes the full-length of the p51A mRNA open reading frame. The base sequence of the former gene is shown in SEQ ID NO: 9 of the sequence listing. In performing the PCR method, a DNA polymerase EX Taq (manufactured by Takara Shuzo) was used, and the PCR condition comprised 30 cycles of amplification with each cycle comprising 95°C for one minute, 55°C for one minute, and 72°C for one minute. PCR amplified products were subjected to phenol treatment and chloroform treatment to remove protein, and then to ethanol precipitation. Then this DNA was washed in 70% ethanol and dissolved in a sterile water. After these purified DNAs and 20 ng of E. coli vector pBluescript KS(+) (manufactured by Toyobo) which was cleaved with a restriction enzyme EcoRV (manufactured by Takara Shuzo), and in which one thymine was added (referred to hereinafter as pBS/EcoRV TA), were ligated using a DNA ligation kit (manufactured by Takara Shuzo) (the plasmid containing the amplified gene obtained with the primer set shown in SEQ ID NO: 5 and 6 is hereinafter referred to as pBS/p51-1, and the gene containing the amplified gene obtained with the primer

set shown in SEQ ID NO: 7 and 8 is hereinafter referred to as pBS/p51A ORF). The absence of mutation in the amplified genes was confirmed by determining the base sequences of pBS/p51-1 and pBS/p51A ORF. The determination of the base sequence was performed using an automated sequencer LONG READIR4200 (manufactured by LICOR). Subsequently, pBS/p51-1 was cleaved with restriction enzymes EcoRV and PstI (manufactured by Toyobo), which were then fractionated on a 0.8% agarose electrophoresis, extracted, and purified. For purification, EASYTRAP (manufactured by Takara Shuzo) was used. This purified DNA was used as a template for the screening probe.

(1-2) Screening of a novel gene fragment encoding the p51 promoter region

Subsequently, the screening of gene fragments of a gene encoding the p51 promoter region from a human genomic library was attempted. As the human genomic library, Easy-to-Handle Eukaryotic Genomic Library from human (manufactured by Mo Bi Tec) was used. The infection efficiency of this library was three million plaque forming units per microliter. Using this library, a membrane for plaque hybridization was prepared in the following manner. 0.02 μl of the library solution and 0.9 ml of C600 E. coli solution (E. coli strain C600 was cultured under shaking in 50 ml of the LB medium (0.5% yeast extract, 1% peptone, 0.5% sodium chloride) containing 0.2% maltose and 10 mM

magnesium sulfate) at 37°C for 16 hours, centrifuged at 5000 rpm for 5 minutes, recovered, and then suspended in 25 ml of 10 mM magnesium sulfate) were mixed, and incubated at 37°C for 15 minutes so as to be infected with phage. Seven ml of LB soft agar (0.7% agar was added to 0.5% yeast extract, 1% peptone, 0.5% sodium chloride and 10 mM magnesium sulfate to prepare an agar medium) that was incubated at 47°C after melting was added thereto, gently mixed, and then was plated onto a LB-magnesium sulfate plate (1.5% agar was added to 0.5% yeast extract, 1% peptone, 0.5% sodium chloride and 10 mM magnesium sulfate to prepare an agar medium) with an internal diameter of 150 mm. By this procedure, 60,000 plaques appeared per plate. A similar procedure was used to plate on a total of 23 plates. They were incubated at 37°C for 16 hours to form plaques. After the plate was cooled at 4°C for one hour, the plaques were adhered to a nylon membrane, Colony plaque screen (manufactured by NEN). After this membrane was dried, it was allowed to denature in 500 ml of an alkaline solution (0.2 N sodium hydroxide, 1.5 M sodium chloride) at room temperature for 2 minutes followed by neutralization in 500 ml of a neutralizing solution (0.5 M Tris-HCl, pH 7.2, 1.5 M sodium chloride, 1 mM ethylenediaminetetraacetic acid disodium salt) at room temperature for 2 minutes. This membrane was then incubated in a 3 X SSC solution (450 mM sodium chloride, 45 mM trisodium citrate) at 55°C for one

hour, and dried to prepare a membrane for plaque hybridization. Fifty ng of the above purified cDNA fragment obtained by cleaving pBS/p51-1 with restriction enzymes EcoRV and PstI was labeled using Prime-It II (manufactured by Stratagene) to prepare a probe for screening.

Then the membrane was used for plaque hybridization. The above membrane was incubated in 50 ml of a hybridization solution (50 mM Tris-HCl, pH 7.5, 1 M sodium chloride, 1% sodium dodecylsulfate, 10% dextran sulfate, 0.2 mg/ml yeast RNA, 0.2 mg/ml salmon sperm DNA) at 65°C for one hour as prehybridization. Then a radioisotope-labeled cDNA fragment was added to one million dpm/ml in terms of the amount of radioisotope and incubated at 65°C for 16 hours as hybridization. Subsequently the membrane was washed in a 2 x SSC solution (300 mM sodium chloride, 30 mM trisodium citrate) containing 0.1% sodium dodecylsulfate at 65°C for 30 minutes. The washing was performed twice. Then, the membrane was subjected to autoradiography to detect positive plaques.

Then, plaque groups containing plaques corresponding to positive signal were isolated, and suspended in one ml of the SM buffer solution (50 mM Tris-HCl, pH 7.5, 100 mM sodium chloride, 10 mM magnesium sulfate, 0.01% gelatin). The solution was incubated at 4°C for 16 hours to elute phage, and the phage was recovered in the supernatant fluid after

centrifuging at 13000 rpm for 10 minutes. The phage solution was plated on a LB-magnesium sulfate plate in a manner similar to the above to screen positive plaques. As a result, positive plaques were successfully isolated as completely isolated plaques. From these plaques, phage was isolated in a manner similar to the above.

Subsequently, the following experiment was performed in order to determine the base sequence of the library DNA contained in this phage. The library has been made using lambda PS phage (manufactured by Mo Bi Tec). The phage has the recombinant site of loxP in the base sequence thereof, and thereby it can be introduced into an E. coli strain BNN132 having Cre recombinase in order to excise the vector portion containing the genome library sequence from the phage. This procedure was carried out as described below. Twenty μl of the phage solution was mixed with 200 μl of the BNN132 E. coli solution (E. coli strain BNN132 was cultured under shaking in 50 ml of the LB medium containing 0.2% maltose and 10 mM magnesium sulfate at 37°C for 16 hours, and then recovered by centrifuging at 5000 rpm for 5 minutes, which was then suspended in 25 ml solution of 10 mM magnesium sulfate) and incubated at 37°C for 30 minutes. The mixture was then plated onto the LB-ampicillin plate (1.5% agar was added to 0.5% yeast extract, 1% peptone, 0.5% sodium chloride and 0.1 mg/ml ampicillin sodium to prepare an

agar medium), and the plasmid of interest was prepared from the colonies appeared. The preparation of the plasmid was performed according to the procedure described in Lab Manual Gene Engineering (Published by Maruzen, edited by Masami Muramatsu, 1990) on pages 53-55. The plasmid was found to contain about 15 kb of the library gene. This is referred to hereinafter as pPS/library.

(1-3) Sequencing of the gene fragment encoding the p51 promoter region

Then, part of the base sequence of the library gene in pPS/library was determined using an automated sequencer LONG READIR4200 to confirm that it contains part of the cDNA sequence complementing p51A mRNA. It was also found that a 0.6 kb gene fragment obtained by cleaving of pPS/library with a restriction enzyme EcoRV contains part of the cDNA sequence that complements p51A mRNA. The pPS/library was then cleaved with a restriction enzyme PvuII (manufactured by Toyobo), fractionated on 0.8% agarose electrophoresis, and blotted on a nylon membrane Hybond N+ (manufactured by Amersham). Blotting of the plasmid fragment was performed according to the procedure described in Lab Manual Human Genome Mapping (published by Maruzen, eddited by Masaaki Hori and Yusuke Nakamura, 1991) on pages 26-36. This membrane was used in Southern blotting according to the following procedure. As a cDNA fragment for probing, pPS/library

was used and cleaved with a restriction enzyme EcoRV, and after fractionation on a 0.8% agarose electrophoresis a 0.6 kb gene fragment was excised and purified using EASYTRAP. This purified cDNA was radiolabeled using Prime-It II to prepare a probe. The membrane was incubated in a hybridization solution for Southern blotting (10% sodium dodecylsulfate, 7% PEG8000) at 65°C for one hour as prehybridization. Then the above probe was added to one million dpm/ml in terms of the amount of radioisotope and incubated at 65°C for 16 hours for hybridization. Subsequently, the membrane was washed in a 2 x SSC solution containing 0.1% sodium dodecylsulfate at 65°C for 30 minutes. The washing was performed twice. Then, the membrane was subjected to autoradiography to demonstrate that a PvuII-cleaved fragment containing the immediate upstream region of p51A mRNA was 5.5 kb in length. Then, pPS/library was cleaved with a restriction enzyme PvuII, and after fractionation on a 0.8% agarose electrophoresis a 5.5 kb gene fragment was excised and purified using EASYTRAP. The purified cDNA fragment was ligated, using the DNA ligation kit, to pBluescript II Sk(+) that had been cleaved with a restriction enzyme EcoRV followed by dephosphorylation with shrimp alkaline phosphatase. The plasmid is referred to hereinafter as pBS/PvuII5.5. Its base sequence was determined using the automated sequencer LONG READIR 4200. The base sequence and the partial base sequence

of the pPS/library determined above were combined to obtain the base sequence as set forth in SEQ ID NO: 2 of the sequence listing. Furthermore, a base sequence obtained by removing the part that encodes the 5'-untranslated region of p51 from the base sequence as set forth in SEQ ID NO: 2 of the sequence listing is shown in SEQ ID NO: 1 of the sequence listing. The 3'-terminal of this PvuII-cleaved fragment was found to be located about 0.22 kb upstream of the base at position 1 of SEQ ID NO: 9 of the sequence listing that is a cDNA sequence corresponding to p51A mRNA.

Accordingly, in order to prepare a plasmid containing bases up to the base at position 1 of SEQ ID NO: 9 of the sequence listing that is cDNA corresponding to part of p51A mRNA, the following procedure was performed. The pPS/library was cleaved with a restriction enzyme EcoRV, which was fractionated on a 0.8% agarose electrophoresis and then a 0.6 kb gene fragment was excised and purified using EASYTRAP, which was ligated using a DNA ligation kit to pBS/PvuII5.5 that had been cleaved with a restriction enzyme EcoRV and a restriction enzyme SmaI (manufactured by Takara Shuzo) followed by dephosphorylation with shrimp alkaline phosphatase. The plasmid obtained was analyzed and the plasmid that was ligated in the correct direction was isolated. The plasmid is referred to hereinafter as pBS/p51 promoter.

In order to demonstrate that the base

sequence is surely present on the genome, the following experiment was performed. From a colon cancer cell line HCT116, genomic DNA was prepared, which was used as a template in a PCR. The genomic DNA was prepared according to Cell Engineering Experimental Protocol (published by Shujunsha, edited by Tokyo University, the Institute of Medical Science, Department of Anti-Cancer Research, 1993) on pages 16-19. The primers used are those shown in SEQ ID NO: 10 and 11. Respectively, they correspond to the sense strand of the bases at positions from 3543 to 3570 and the antisense strand of the bases at positions from 5458 to 5487 of the base sequence as set forth in SEQ ID NO: 1 of the sequence listing. Polymerase used was LA Taq (manufactured by Takara Shuzo), and PCR comprised 30 cycles of amplification with each cycle comprising 94°C for one minute and 68°C for three minutes. The PCR products were subjected to a 0.8% agarose electrophoresis to confirm that a gene fragment of 1.9 kb was specifically amplified. Furthermore, after the fragment was excised, it was purified using EASYTRAP, and ligated to pBS/EcoRV TA using a DNA ligation kit. The base sequence of this plasmid was determined using an automated sequencer LONG READIR 4200, and it was confirmed to completely match the base sequence in the pBS/p51 promoter.

(1-4) Construction of a luciferase vector containing the p51 promoter region

In order to confirm that promoter activity is present on the isolated region, a vector in which the gene sequence was ligated upstream of the luciferase reporter gene was prepared according to the following method. As the vector having the luciferase reporter gene, pGL2 (manufactured by Promega) was used. First, to allow for integration of pGL2 and selection in mammalian cells, and a neomycin-resistant gene was introduced into this vector. The pGL2 cleaved with a restriction enzyme BamHI (manufactured by Toyobo) was ligated using a DNA ligation kit to a 2617-base neomycin-resistant gene, being obtained by cleaving pMAM neo (manufactured by Toyobo) with BamHI and fractionating on a 0.8% agarose gel electrophoresis excised and purified using EASYTRAP. The plasmid is referred to hereinafter as pGL2-neo. Subsequently, pGL2-neo was cleaved with a restriction enzyme XhoI (manufactured by Toyobo), precipitated with ethanol, washed in 70% ethanol, and dissolved in purified water. The cleaved end of this DNA was blunt-ended using a DNA blunting kit (manufactured by Takara Shuzo), precipitated with ethanol, washed in 70% ethanol, and dissolved in purified water. Then, this DNA was cleaved with a restriction enzyme KpnI (manufactured by Boehringer Mannheim), treated with phenol, treated with chloroform to remove protein, precipitated with

ethanol, washed in 70% ethanol, and dissolved in sterile water. This DNA is referred to hereinafter as pGL2-neo/XhoI (blunting), KpnI.

Subsequently, the pBS/p51 promoter was cleaved with a restriction enzyme NotI (manufactured by Boehringer Mannheim), precipitated with ethanol, washed in 70% ethanol, and dissolved in purified water. After the cleaved end of this DNA was blunt-ended using a DNA blunting kit (manufactured by Takara Shuzo), precipitated with ethanol, washed in 70% ethanol, and dissolved in purified water. Then, this DNA was cleaved with a restriction enzyme KpnI (manufactured by Boehringer Mannheim), fractionated on a 0.8% agarose electrophoresis, and a 5.7 kb gene fragment was excised and purified using EASYTRAP. This gene fragment is referred to hereinafter as pBS/p51 promoter/NotI (blunting), KpnI5.7. Then, pGL2-neo/XhoI (blunting), KpnI and pBS/p51 promoter/NotI (blunting), KpnI5.7 were ligated using a DNA ligation kit. This plasmid is referred to hereinafter as pGL2-neo/p51 promoter. The gene sequence of this plasmid is shown in SEQ ID NO: 12 of the sequence listing. The restriction map of this plasmid is shown in Figure 1.

(1-5) Effect of various drugs on the amount of p51 transcribed

1) Northern hybridization

A variety of stimulations including stimulation with a drug were applied to a cell line HCT116

derived from colon cancer to investigate changes in the amount expressed of p51 by Northern blotting. 200,000 cells of HCT116 cells were plated on a culture dish with a diameter of 60 mm, and cultured in the presence of 5% carbon dioxide at 37°C for two days. The medium used was McCOY's 5A (manufactured by Nisseiken) to which fetal bovine serum (FBS) was added to a final concentration of 10% (referred to hereinafter as McCOY's 5A/10% FBS). Then it was treated with A23187 (0.1 μM, 1 μM), cisplatin (1 μg/ml, 10 μg/ml), CPTcAMP (0.1 μM, 1 μM), etoposide (10 ng/ml, 0.1 μg/ml), genistein (1 μM, 10 μM), ML-236B (1 μg/ml, 10 μg/ml), milurinone (1 μM, 10 μM), mitomycin C (5 μg/ml, 50 μg/ml), NKH477 (0.1 μM, 1 μM), okadaic acid (1 nM, 10 nM), radicicol (0.1 μg/ml, 1 μg/ml), staurosporin (1 nM, 10 nM), taxol (1 μg/ml, 10 μg/ml), trichostatin A (0.1 μg/ml, 1 μg/ml), radiation (50 J/m^2, 100 J/m^2), vitamin D_3 (10 nM, 100 nM), vincristine (1 μg/ml, 10 μg/ml), and wortmannin (0.1 μM, 1 μM), and further cultured in the presence of 5% carbon dioxide at 37°C for 24 hours. Then, from the cells, RNA was prepared using ISOGEN (manufactured by Nippongene). 20 μg each of RNA was fractionated on a 1% agarose electrophoresis containing formamldehyde, and blotted on a nylon membrane HybondN+ according to the method in the above New Cell Engineering Experimental Protocol on pages 194-197. As the probe, pBS/p51A ORF was cleaved with a restriction enzyme EcoRI (manufactured by Toyobo),

fractionated on a 0.8% agarose electrophoresis, and then a 0.6 kb gene fragment was excised and purified using EASYTRAP, which was radiolabeled with Prime-It II and used. The membrane was incubated in 10 ml of a prehybridization solution (25 mM phosphate buffer solution, pH 7.0, 6 X SSC, 50% formamide, 5 X Denhardt's solution, 0.1% sodium dodecylsulfate, 0.2 mg/ml salmon sperm DNA) at 42°C for four hours as prehybridization. Then it was replaced with 10 ml of a hybridization solution (20 mM phosphate buffer solution, pH 7.0, 6 X SSC, 50% formamide, 1 X Denhardt's solution, 0.1% sodium dodecylsulfate, 0.1 mg/ml salmon sperm DNA, 4% dextran sulfate), and then the above radiolabeled probe was added to one million dpm/ml and incubated at 42°C for 16 hours as hybridization. Subsequently, it was washed in a 2 x SSC solution containing 0.1% sodium dodecylsulfate at 65°C for 30 minutes. The washing was performed twice. Then, the membrane was analyzed on autoradiography.

2) Result

As a result, a specific band that reacts with p51A-derived cDNA was only observed in the cells treated with 1 μg/ml trichostatin A. In any other stimulation, no increases in the amount of p51A mRNA were observed. Figure 2 shows an increase in the amount of p51A mRNA with trichostatin A treatment. Figure 2 shows blotting of 20 μg of RNA from: lane 1 and 2: cells treated with taxol (1 μg/ml, 10 μg/ml),

lane 3 and 4: cells treated with trichostatin A (0.1 μg/ml, 1 μg/ml), lane 5 and 6: cells treated with radiation (50 J/m^2, 100 J/m^2), lane 7 and 8: cells treated with vitamin D_3 (10 nM, 100 nM), lane 9 and 10: cells treated with vincristine (1 μg/ml, 10 μg/ml), and lane 11 and 12: cells treated with wortmannin (0.1 μM, 1 μM).

(1-6) Functional analysis of the p51 promoter region

Subsequently, the following experiment was carried out in order to perform functional analysis of the p51 promoter region. 200,000 cells of HCT116 were plated on a culture dish with a diameter of 60 mm. The medium used was McCOY's 5A/10% FBS. These were cultured in the presence of 5% carbon dioxide at 37°C for 48 hours. The medium was replaced with McCOY's 5A containing no serum. Subsequently, 2 μg each of pGL2-neo/p51 promoter or pGL2-neo vector was introduced. The plasmid was introduced using the lipofectamine plus reagent (manufactured by GIBCO BRL). Thus, 125 μl of McCOY's 5A and 8 μl of the plus solution were added to the plasmid solution, stirred well, and then incubated at room temperature for 15 minutes. To this solution, 125 μl of McCOY's 5A and 12 μl of lipofectamine solution were added, stirred well, and then incubated at room temperature for further 15 minutes. This was added to the HCT116 cells incubated by the above procedure, gently mixed, and cultured in the presence of 5% carbon dioxide at 37°C for three hours to

introduce plasmid. Subsequently, the medium was replaced with McCOY's 5A/10% FBS, and cultured in the presence of 5% carbon dioxide at 37°C for 21 hours. Then, trichostatin A was added to two groups of cells to a final concentration of 0 or 1 μg/ml, and cultured in the presence of 5% carbon dioxide at 37°C for 24 hours. For these cells, the luciferase activity was determined to investigate promoter activity. As a negative control, cells containing no luciferase gene, pcDNA3/lacZ (the lacZ gene ligated to the mammalian cell expression vector pcDNA3) was transfected in a procedure similar to the above. The luciferase activity was determined according to the following method.

The medium in the cell culture dish was removed, and 5 ml of the cell lysis reagent (25 mM Tris-HCl, pH 7.8, 2 mM dithiothreitol, 2 mM CDTA, 0.2% triton X-100, 10% glycerol) was added and incubated at room temperature for 15 minutes. The cell lysis solution was taken at 10, 20, 30, 40, and 50 μl aliquotes, to which 50 μl of the substrate solution (20 mM Tricine-sodium hydroxide, pH 7.8, 1.07 mM basic magnesium carbonate, 2.67 mM magnesium sulfate, 0.1 mM ethylenediaminetetraacetic acid disodium salt, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferine, 530 μM adenosine triphosphate) was added, and incubated at room temperature for 15 seconds, and its chemi-luminescence intensity was determined using a

luminometer LUMINOSKAN (manufactured by Dainippon Pharmaceutical). The result is shown in Figure 3.

These results demonstrated that pGL2-neo/p51 promoter-introduced cell lysis solution exhibits a significantly high fluorescence intensity compared to pGL2-neo. The administration of trichostatin A also markedly enhanced fluorescence intensity in the pGL2-neo/p51 promoter. The result is in agreement with the above result in Figure 2.

The above experimental data demonstrated that the isolated gene fragment is a functional p51 promoter region.

Example 2

Screening of drugs that modify the p51 promoter function

(2-1) Preparation of a cultured cell line for screening

The cultured cell line retaining the pGL2-neo/p51 promoter plasmid can be used as cells for screening drugs that modify the p51 promoter function. Said drug is effective for treatment of various diseases including cancer. Said cells were prepared according to the following procedure.

As the plasmid-introduced cells, the HCT116 cell line was used. 500,000 cells of HCT116 cell were plated on a cuture dish with a diameter of 60 mm. The medium used was McCOY's 5A/10% FBS. These were cultured in the presence of 5% carbon dioxide at 37°C

for 48 hours. The medium was replaced with McCOY's 5A containing no serum. As the plasmid for introduction, two μg of pGL2-neo/p51 promoter was dissolved in 4 μl of the TE solution to prepare a plasmid solution. The plasmid was introduced using the lipofectamine plus reagent (manufactured by GIBCO BRL). Thus, 125 μl of McCOY's 5A and 8 μl of the plus solution were added to the plasmid solution, stirred well, and then incubated at room temperature for 15 minutes. To this solution, 125 μl of McCOY's 5A and 12 μl of the lipofectamine solution were added, stirred well, and then incubated at room temperature for further 15 minutes. This was added to the HCT116 cells cultured by the above procedure, gently mixed, and incubated in the presence of 5% carbon dioxide at 37°C for three hours to permit the introduction of the plasmid. Subsequently, the medium was replaced with McCOY's 5A/10% FBS, and incubated in the presence of 5% carbon dioxide at 37°C for 21 hours. Then, the cells were recovered from the culture dish with PBS containing 0.25% trypsin and 0.02% EDTA, and 1% amount thereof was plated onto a culture dish with a diameter of 100 mm. It was further cultured in McCOY's 5A/10% FBS medium in the presence of 5% carbon dioxide at 37°C for further 72 hours, and then 1.2 mg/ml of geneticin (manufactured by Nacalai Tesque) was added as a selection drug. By replacing the medium every three days, and further culturing in the above condition for further two weeks, the cells

into whose genes the plasmid gene was introduced, and became geneticin-resistant, were selected. Subsequently, 50 clones were isolated from the cell group that formed colonies on the dish, and were plated on a 24-well culture dish to continue being cultured.

These clones are expected to exhibit an enhanced luciferase activity to stimulation such as the activation of the p51 promoter function. Thus, the reactivity of these clones to trichostatin A was investigated by the following procedure. 500,000 cells each of the cloned cells were plated on a cuture dish with a diameter of 60 mm. The medium used was McCOY's 5A/10% FBS. These were cultured in the presence of 5% carbon dioxide at 37°C for 48 hours. Then, trichostatin A was added to the two groups of cells to a final concentration of 0 or 0.1 μg/ml, and cultured in the presence of 5% carbon dioxide at 37°C for 24 hours. For these cells, their luciferase activity was determined to examine the effect on the p51 promoter activity. The luciferase activity was determined according to the above method. As a result, several clones were obtained that exhibit a significantly enhanced luciferase activity to 0.1 μg/ml trichostatin A, from among which HCT116/p51 promoter clone #9 was selected as the cell for screening. The result of luciferase assay is shown in Table 1.

Table 1

Clone No.	#5	#9	#17	#18
Without TsA	3.9	1.0	17.0	6.8
With TsA	29.5	33.3	44.1	14.0

In the Table, TsA represents trichostatin A. The trichostatin A treatment group received at 0.1 μg/ml. Values in the table show the measured values of luciferase activity.

(2-2) Screening of drugs that modify the p51 promoter function

HCT116/p51 promoter clone #9 cultured cell line is useful for screening drugs that induce the activation of the p51 promoter function. Said screening was performed according to the following procedure.

10,000 cells of HCT116/p51 promoter clone #9 cultured cell line were plated onto a 96-well culture dish, and cultured in 200 μl of RPMI1640/10% FBS in the presence of 5% carbon dioxide at 37°C for 24 hours. Subsequently, a drug for screening was added, culturing was continued for 24 hours, and the luciferase activity was determined. As the drug, synthetic compounds or microbial secondary metabolites were used.

1200 compounds and 9600 microbial secondary metabolites were screened and 71 samples that is capable of activating the p51 promoter activity at a

level equal to or greater than that of 0.1 μg/ml trichostatin A. Substances that activate the p51 promoter are expected to become therapeutic agents for treating diseases including cancer.

INDUSTRIAL APPLICABILITY

In accordance with the present invention, there are provided a gene encoding the promoter region of protein p51 which is capable of inducing cell death, and a gene encoding the 5'-untranslated region of p51. They are useful in diagnosing and treating diseases including cancer caused by abnormal regulation of cell propagation. Furthermore, in accordance with the present invention, there are provided the antisense DNAs and the antisense RNAs of these genes, nucleic acid probes comprising parts or all of these genes, methods of detecting the above genes of the present invention or analogous genes using said nucleic acid probes, transformants in which the above gene of the present invention has been introduced, and methods of screening drugs using them. These genes are also useful in diagnosing and treating diseases including cancer, etc.

SEQUENCE LISTING FREE TEXT

The base sequences of SEQ ID NO: 5-8, 10 and 11 of the sequence listing show PCR primers.

The base sequence of SEQ ID NO: 9 of the

sequence listing shows the 5'-untranslated region of p51A mRNA and encodes 165 bases at the 5'-end of the open reading frame of p51A mRNA.

The base sequences of SEQ ID NO: 12 of the sequence listing shows the DNA sequence of a plasmid containing the p51 promoter and the neomycin-resistant gene.

CLAIMS

1. A gene encoding the p51 promoter region shown in the following (1), (2), (3), (4), (5), or (6):

(1) DNA that encodes the p51 promoter region having the base sequence as set forth in SEQ ID NO: 1 of the sequence listing;

(2) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in the base sequence as set forth in SEQ ID NO: 1 of the sequence listing, and that has p51 promoter activity;

(3) DNA that hybridizes to the base sequence as set forth in SEQ ID NO: 1 of the sequence listing under a stringent condition, and that has p51 promoter activity;

(4) DNA that has the base sequence as set forth in SEQ ID NO: 2 of the sequence listing and that encodes the p51 promoter region and the 5'-untranslated region of p51;

(5) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing, and that has p51 promoter activity; and

(6) DNA that hybridizes to the base sequence as set forth in SEQ ID NO: 2 of the sequence listing under a stringent condition, and that has p51 promoter activity.

2. A gene encoding the 5'-untranslated region of p51 shown in the following (7), (8), or (9):

(7) DNA that has a base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing;

(8) DNA that has a base sequence in which one or a plurality of bases have been deleted, substituted, or added in a base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing, and that has a function similar to that of DNA in the above (7); and

(9) DNA that hybridizes to DNA comprising the base sequence of positions from 5677 to 5960 in the base sequence as set forth in SEQ ID NO: 2 of the sequence listing under a stringent condition, and that has a function similar to that of DNA in the above (7).

3. A recombinant plasmid comprising the gene of claim 1.

4. A transformant or a transductant comprising the recombinant plasmid of claim 3.

5. A nucleic acid probe comprising all or parts of the gene of claim 1 or 2.

6. A method of cloning the p51 promoter region or an analogous gene using the nucleic acid probe of claim 5.

7. A DNA sequence that is an antisense to all or parts of the gene of claim 1 or 2 and that enhances or inhibits the expression of p51 gene.

8. A RNA sequence that is an antisense to all or parts of the gene of claim 1 or 2 and that enhances or inhibits the expression of the p51 gene.

9. A method of screening drugs that act on p51 promoter using the transformant or the transductant of claim 4.

10. A compound that enhances or inhibits the expression of the p51 gene, said compound being selected by the screening method of claim 9.

11. A pharmaceutical formulation comprising the DNA sequence of claim 1, the DNA sequence of claim 7, or the RNA sequence of claim 8.

FIG. 1

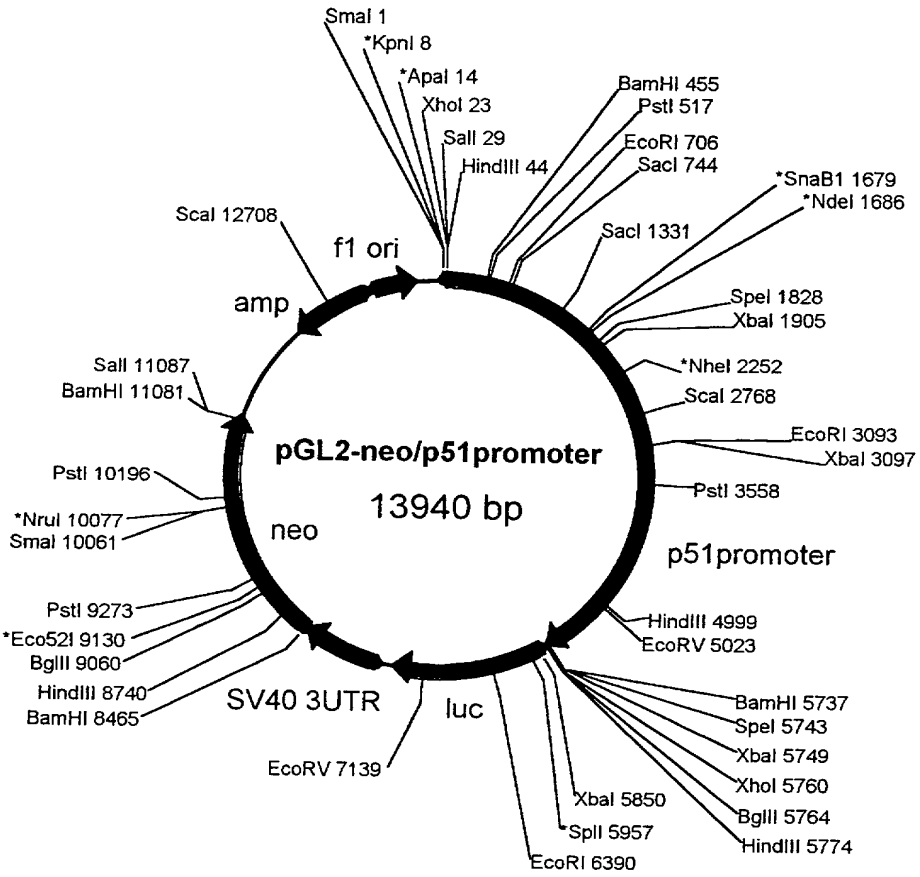


FIG. 2

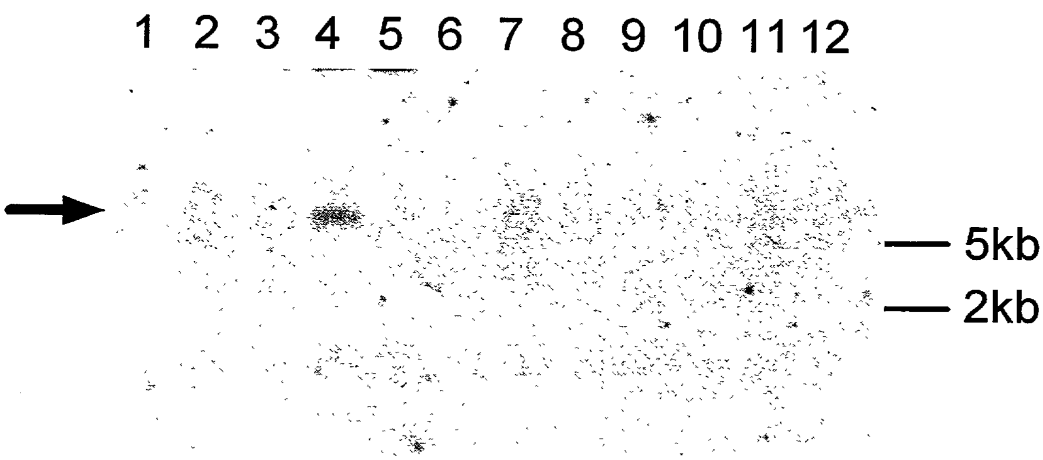
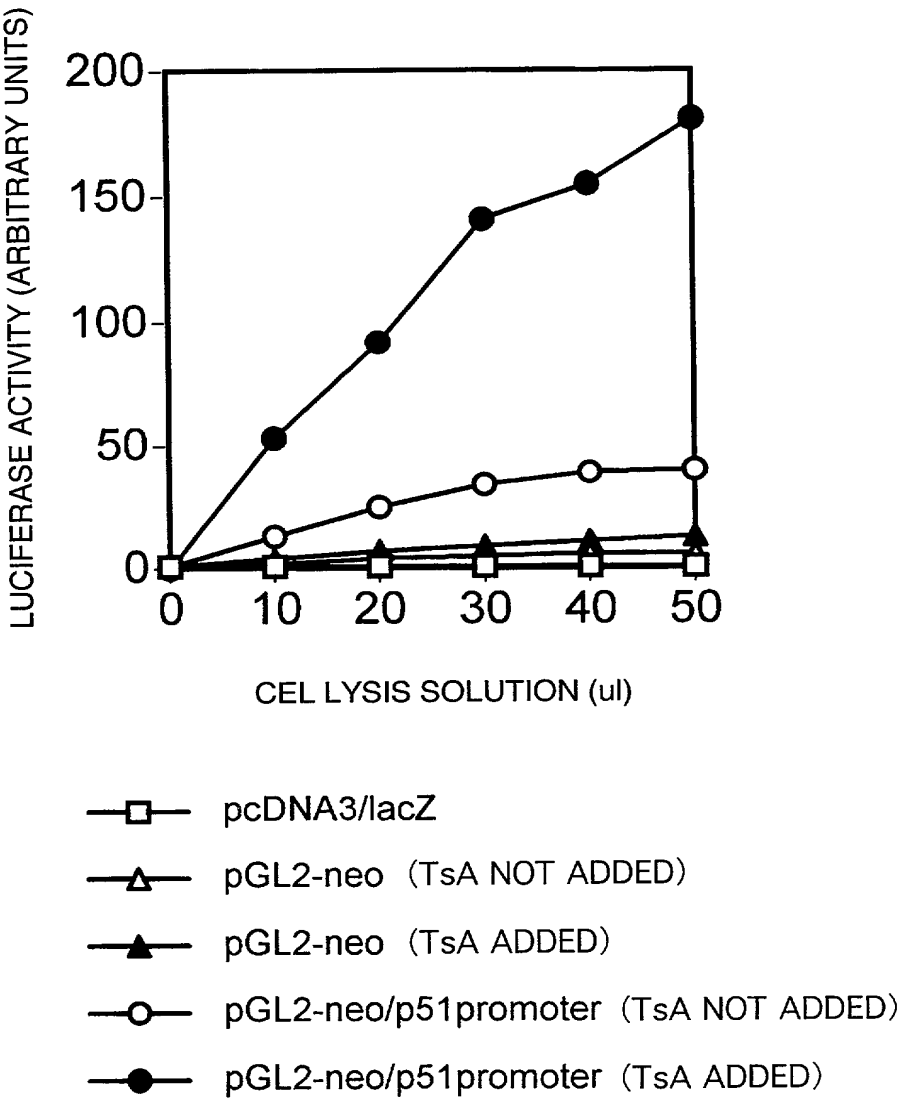


FIG. 3


LUCIFERASE ACTIVITY (ARBITRARY UNITS)
200
150
100
50
0
0
10
20
30
40
50
CEL LYSIS SOLUTION (ul)
pcDNA3/lacZ
pGL2-neo (TsA NOT ADDED)
pGL2-neo (TsA ADDED)
pGL2-neo/p51promoter (TsA NOT ADDED)
pGL2-neo/p51promoter (TsA ADDED)

E595-01 #1

COMBINED DECLARATION AND POWER OF ATTORNEY
(宣誓書及び委任状)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

"GENE ENCODING PROMOTER REGION OF TUMOR SUPPRESSOR GENE P51 AND USE THEREOF"

the specification of which: (check one) ☐ is attached hereto.

☒ was filed on June 28, 2000
as Application Serial No. PCT/JP00/04261
and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended, by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me which is material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date earlier than that of the application(s) on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
11-183195 (Number)	Japan (Country)	June 29, 1999 (Day/Month/Year Filed)	☒ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No

I hereby claim the benefit under Title 35, United States Code, 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to discolse to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international fiiling date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

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I hereby appoint the following as my attorneys of record with full power of substitution and revocation to prosecute this application and to transact all business in the Patent and Trademark Office:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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